

Follistatin-Related Protein and Follistatin Differentially Neutralize Endogenous *vs.* Exogenous Activin

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Follistatin-related protein (FSRP) is a new addition to the expanding follistatin (FS)-related gene family whose members contain at least one conserved 10-cysteine follistatin domain. In contrast to other members of this family, FSRP and follistatin also share a common exon/intron domain structure, substantial primary sequence homology, and an ability to irreversibly bind activin. In this study, we further explored the hypothesis that FSRP is a functional as well as structural homologue of FS. N-terminal sequencing of recombinant FSRP revealed that signal peptide cleavage occurs within exon 1, a significant structural difference from FS, in which cleavage occurs at the exon/intron boundary. Solid-phase radioligand and competition assays revealed both FS and FSRP to preferentially bind activin with the next closest TGF- β superfamily member, bone-morphogenic protein-7, being at least 500-fold less potent. Consistent with their similar activin-binding affinities, FSRP and FS both prevented exogenous

(endocrine or paracrine) activin from accessing its receptor and inducing gene transcription in bioassays. However, FS was at least 100-fold more potent than FSRP in inhibiting gene transcription and FSH release mediated by endogenously produced (autocrine) activin-A or activin-B in multiple cell systems. Finally, FSRP lacks the heparin-binding sequence found in FS, and we found that it was also unable to bind cell surface heparin sulfated proteoglycans. These findings suggest that structural differences between FSRP and FS may underlie their different neutralizing capabilities with respect to exogenous *vs.* endogenous activin. Taken together with our previous studies showing that activin binding is essential for FS's biological activity, the differential activities of FSRP and FS further indicate that activin binding is necessary but not sufficient to account for all of FS's actions. (*Endocrinology* 143: 1613–1624, 2002)

FOLLISTATIN (FS) (1) is a monomeric glycoprotein first isolated from ovarian follicular fluid on the basis of its ability to suppress FSH secretion by pituitary cells *in vitro* (1, 2). FS has subsequently been shown to bind activin selectively, nearly irreversibly, and with high affinity (3, 4), rendering it biologically inactive (5) and prone to endocytotic degradation (6).

The effects of activin, an important regulator of developmental and homeostatic mechanisms in multiple organ systems (7, 8), are frequently modified in a paracrine/autocrine manner by coordinate expression of FS (9). For example, FS is produced within the pituitary to locally modulate activin's stimulatory effect on FSH release (10–12). In the ovary, FS modulates such activin effects as oocyte germinal vesicle breakdown and granulosa cell steroidogenesis (13–15). During cartilage maturation and osteoclast formation in endochondral bone development, FS expression varies temporally to precisely regulate activin's signaling capacity (16–18). FS has also been implicated as a modulator of activin's effects on mesoderm induction (19, 20), prostatic epithelial cell growth (21), pancreatic islet cell function (22), hepatocellular regeneration (23), and placental function during par-

turition (24). Therefore, the local regulation of activin's bioavailability has broad physiological significance.

A domain structure for FS has been proposed based on the discovery that its exons encode highly conserved amino acid sequences enriched in cysteine residues (2, 25). The N-terminal domain of FS appears to be the site responsible for the majority of its activin-binding ability, with tryptophan residues at positions 4 and 36 being especially important in this capacity (25, 26). This domain is followed by a series of three consecutive FS domains, which are encoded by separate exons and are distinguished by their conserved alignment of 10 cysteine residues. Presumably arising from exon shuffling, these FS domains have also been identified in several extracellular matrix proteins collectively referred to as FS-related genes, including agrin, testican, and SPARC (secreted protein acidic and rich in cysteines) (27–29). Importantly, the first FS domain within FS contains a lysine-rich heparin binding sequence that allows FS to associate with cell surface heparin sulfated proteoglycans (30), thereby forming a barrier that can prevent activin from accessing its receptor and inducing specific gene transcription (31, 32).

Follistatin-related protein (FSRP) is a recently described member of the growing FS-related gene family that was originally cloned from a B-cell leukemia line and was called follistatin-related gene based on primary sequence homology to FS (33). Further analysis of this protein revealed an extraordinary level of homology with FS, significantly greater than that manifested by all previously identified

Abbreviations: ACTRII, Activin receptor type II; BMPs, bone-morphogenic proteins; FS, follistatin; FSRP, follistatin-related protein; MIS, müllerian-inhibiting substance; pARE-GFP-lux, plasmid with activin responsive element, green fluorescent protein, luciferase; pFAST-1, plasmid with forkhead activin stimulator.

folliculin-related genes, which extended to exon/intron arrangement and overall domain structure (Fig. 1). To reduce confusion within this family, we have referred to this gene product as FSRP (34–36). In addition to its structural homology with FS, FSRP also preferentially binds and neutralizes activin-A with greater potency, compared with bone-morphogenetic protein (BMP)-2, -6, and -7 (35, 36). Like FS, FSRP's activin binding is rapid and nearly irreversible, but the affinity of FSRP for activin is 2.4-fold lower than that observed for FS (35). These observations suggest that, like FS, FSRP may also serve to effectively limit the bioavailability of activin, perhaps acting at different stages of development or within different tissues.

On the other hand, several intriguing differences between FS and FSRP suggest that these activin-binding proteins may not be complete functional homologs. Although FSRP and FS are ubiquitously expressed, they are maximally expressed in different tissues (33, 35, 36). In addition, we have recently demonstrated that FSRP, unlike FS, is localized to the nucleus in many cell types and appears to be secreted only by those cells that have the highest levels of FSRP transcription (35).

To further explore their functional differences, we used

highly purified preparations of recombinant human FSRP and FS288 to more precisely compare their ligand-binding specificity among different TGF- β superfamily members. We then examined FSRP and FS for inhibition of activin's actions when activin was included in the treatments (exogenous) and activin was derived from the cells themselves (endogenous). Despite their similar structure, ligand-binding specificity, and ability to neutralize exogenous activin, FSRP was nearly 100-fold less potent than FS in its ability to neutralize endogenous activin. Taken together with our previous observation that FSRP is both a secreted and nuclear protein, our results suggest that FSRP is not simply a functional homologue of FS and likely has unique biological actions.

Materials and Methods

Materials

The FSRP cDNA was tagged at the C terminus with either FLAG or human Fc and was then expressed in 293 cells. Tagged FSRP was purified by immunoaffinity chromatography using anti-FLAG M2 affinity gel or protein A resin, respectively. Eluted fractions were neutralized, pooled, and dialyzed against PBS, pH 7.4. By Coomassie-stained SDS-PAGE gels, the purities of the proteins were estimated to be more than

SIGNAL DOMAIN

hFS315	-29	MVRARHQPGGLCLLLLLLCQFMEDRSAQA	-1
hFSRP	-26	MRPGAGPLWPLPWGALAWAVGFVSS	-1

N-TERMINAL DOMAIN

hFS315	1	GN ^C WLRQAKNGR ^C QVLYKTELSKEE ^{CC} STGRLSTSWTEEDVNDNTLFKWMIFNGGAPN ^C IP ^C CK	63
hFSRP	1	MGSGNPAPGGV ^C WLQGGQ ^{CC} ATCSLVLTQDVTRAE ^{CC} ASGNIDTAWSNLTHPGNKIN-LLGFLGLVH- ^C LP ^C CK	71
<i>N-terminal extension identified by sequencing</i>			

FOLLISTATIN DOMAIN 1

		<i>heparin binding motif</i>	
hFS315	64	ET ^C ENV ^D C ^G PGKK ^C RMNKKNKPR ^C V ^C AP ^D CSNITWKG ^{PV} CGLDGKTYRNE ^C ALLKAR ^C KEQPELEVQYQGR ^C CK	136
hFSRP	72	DS ^C DGVE ^C GP ^G GKA ^C RMLGGR-PR ^C ECAP ^D CSGLPARLQV ^C GS ^D GATYRDE ^C ELRAAR ^C RGHPDLSVMYRGR ^C CR	143

FOLLISTATIN DOMAIN 2

hFS315	137	KT ^C RDVF ^C PGSST ^C VVDQTNNAY ^C VT ^C CNRI- ^C PEPASSEQYL ^C GN ^D GV ^T YSSA ^C HLRKAT ^C LLGRSIGLAYEGK ^C CI	211
hFSRP	144	KS ^C EHVV ^C PRPQS ^C VVDQTGSAH ^C VV ^C CRAAP ^C PVPSSPGQEL ^C GNNNV ^T YISS ^C HMRQAT ^C FLGRSIGVRHAGS ^C CA	219

FOLLISTATIN DOMAIN 3

hFS315	212	KAKS ^C EDIQ ^C TGGKK ^C CLWDFKVGGR ^C CSL ^C DEL ^C PDSKSD ^E FPV ^C CASDNATYASE ^C AMEKAA ^C SSGVLLLEV ^C KHSGS ^C CN	288
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C-TERMINAL DOMAIN

hFS315	289	SISEDTEEEEEDEDQDYSPFISSILEW	315
hFSRP	220	GTPEPPPGGESAEENFV	229

FIG. 1. Domain structures of FS and FSRP. Both FS and FSRP have a signal peptide, an N-terminal domain, follistatin domains, and a C-terminal domain. Each domain in FS is encoded by its own exon. The same is true for FSRP with the exception of its N-terminal domain. N-terminal sequencing identified the cleavage site of FSRP's signal peptide nine residues before the exon/intron junction of exon 1. Therefore, FSRP's N-domain has a nine-residue extension from exon 1 added to the beginning of exon 2.

90%. To determine the exact site of signal peptide cleavage, we performed 13 cycles of N-terminal sequencing of purified recombinant FSRP-Fc by automated Edman degradation in the MGH Protein Core Facility.

The FSRP antibody was raised in rabbits to purified FSRP-Fc protein. High titer bleeds were pooled and purified by protein A affinity chromatography. Recombinant FS288 and FSH RIA reagents were obtained from the National Hormone and Pituitary Program, NIDDK, and NICHD, NIH. Activin-A, inhibin-A, BMP-4, BMP-6, BMP-7, and TGF- β 1 were obtained from R&D Systems (Minneapolis, MN). Mullerian-inhibiting substance (MIS) was a gift from Dr. David MacLaughlin (Pediatric Surgery, MGH). L β T2 cells were kindly provided by Dr. Pamela L. Mellon (Department of Reproductive Medicine, University of California, San Diego, CA). The human activin receptor type II (ActRII)A vector was kindly provided by Dr. Lawrence Matthews (University of Michigan, Ann Arbor, MI).

Competitive ligand-binding assay

Binding studies were conducted in 96-well microtiter plates that were coated with 25 ng FSRP-Fc or FS288 in 100 μ l 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 25 C on an orbital shaker. Unbound protein was removed by washing three times for 5 min with 200 μ l wash buffer (0.01% Tween in 10 mM PBS solution). Then 200 μ l blocking solution (3% BSA, 0.01% Tween in 10 mM PBS) was added to each well, and the trays were incubated at 25 C for 2 h on an orbital shaker. The wells were then rinsed three times with wash buffer. After blocking, unlabeled activin-A standard or other competitors were preincubated for 1 h in 100 μ l assay buffer (0.01 M PBS, 0.1% gelatin, 0.05% Tween). Radiolabeled activin-A (100,000 cpm), prepared by lactoperoxidase method as previously described (37), was then added in 50 μ l and the reaction continued for an additional hour. This nonequilibrium assay format was used to compensate for the heavily favored and nearly irreversible activin binding that would have displaced competitors if the assay had continued for the usual 2 h. At the end of the 1-h incubation, the wells were washed and counted. The standard curves were analyzed using the NIH RIA computer program that was also used to determine the activity of the competitors.

Activin receptor-binding assay

HEK-293 cells or COS-7 cells were plated in DMEM/F12 media containing 10% FBS into 12-well trays. After 24 h, the cells were transfected with 3 μ g hActRIIA cDNA using Effectene (QIAGEN, Valencia, CA). At 36 h post transfection, the media was aspirated and replaced with fresh media containing approximately 30,000 cpm of 125 I-activin/ml that had been allowed to form complexes with either FS288 or FSRP-Fc at varying concentrations (0.018 nM to 18 nM) for 16 h at 4 C. Nonspecific binding was determined by preincubating the radiolabeled activin-A with 100-fold excess unlabeled activin-A. In addition, as at least FS288 has the known ability to adhere to cells by virtue of its heparin-binding sequence (30), all cell treatments were done in the presence of 5 μ g/ml heparan sulfate to eliminate background binding. The cells were incubated with the activin complexes for 4 h at room temperature. The media were then aspirated, the cells were gently washed with PBS, and the cells were lysed with 1 M NaOH. Lysates were then counted in a γ counter.

HEK-293 cell activin-luciferase reporter system

The plasmid with activin responsive element, green fluorescent protein (pARE-GL3) was constructed by inserting six repeats of the activin response element from the *Xenopus* mix2 gene upstream of the SV40 basal promoter of pARE-GL3 (Promega Corp., Madison, WI) as previously described in detail (32). The *Xenopus* transcription factor FAST-1 (plasmid with forkhead activin stimulator, pFAST-1), which binds to this element, was cotransfected to obtain activin-stimulated reporter activation and was kindly provided by Dr. Malcolm Whitman (Harvard Medical School). The inhibin/activin- β A and - β B cDNAs were kindly provided by Genentech, Inc. (South San Francisco, CA) and use a cytomegalovirus promoter to drive expression. The Renilla luciferase reporter (Promega Corp.) was used as internal transfection control.

HEK 293 cells were maintained in RPMI 1640 medium containing 10% FBS (Life Technologies, Inc., Rockville, MD). To examine the effect of

FSRP or FS on endogenous activin, transfections were performed in 24-well trays using Effectene with a total of 200 ng DNA (80 ng pARE-GL3, 100 ng pFAST-1, 19 ng pRL-TK, and 1 ng of either pINH β A or pINH β B cDNA). To analyze regulation of exogenous activin activity, the β A and β B cDNAs were replaced with empty pcDNA3.

Approximately 16 h after transfection, the media was replaced with RPMI supplemented with 1% FBS (and 0.3 nM activin-A for experiments examining exogenous activin) that had been preincubated for 1 h at 25 C with FS288 (ranging from 0.006 nM to 3.9 nM) or FSRP-Fc (ranging from 0.016 nM to 24.2 nM). After treating for 24 h, the cells were lysed and assayed for luciferase activity using the dual luciferase reporter assay kit (Promega Corp.). Interwell variations in transfection efficiency were corrected by normalizing to Renilla luciferase.

Rat anterior pituitary bioassay

The anterior pituitary glands of adult male Sprague Dawley rats were mechanically and enzymatically dispersed with 0.4% trypsin and 0.25% DNase and then nylon mesh filtered to remove cellular debris. The cells were then plated at 250,000 cells/well in 48-well tissue culture trays in α MEM containing 21 mM NaHCO₃, 10% heat-inactivated FBS, and 10% penicillin/streptomycin solution, pH 7.4. Cells were incubated at 37 C in 95% air, 5% CO₂ for 72 h, after which time the monolayers were washed with PBS and then reincubated in fresh media containing no treatment or various concentrations of pure recombinant human FS288, FSRP-FLAG, or FSRP-Fc. In addition, to examine the relative ability of FS and FSRP-Fc to modulate exogenous *vs.* endogenous activin, these assays were conducted in the presence or absence of exogenous activin-A. To prepare treatments, FSRP or FS was added to medium containing no activin, or 0.35 nM activin-A, serially diluted in the same medium and allowed to stand for 1 h before treating cells. After 72 h, the conditioned media were assayed for rat FSH. For this assay, purified rat FSH was iodinated using chloramine-T and purified from free iodine by PAGE (38). The assay consisted of 100 μ l antibody, 50 μ l radioligand (30,000 cpm), and 150 μ l assay buffer (0.01 M PBS, 0.1% gelatin, and 0.05% Tween), sample or standard. The antirat FSH antibody (FSH S-11) was diluted in 1:400 normal rabbit serum to a final dilution of 1:125,000 that bound 30% of radiolabeled rFSH (NIDDK I-9). The rat FSH RP-2 standard was used to calibrate the assay.

L β T2 cell activin-luciferase reporter system

L β T2 cells, derived from mouse anterior pituitary tumors, have been previously shown to produce abundant activin- β B message as well as FSH β subunit mRNA and FSH protein in response to exogenous activin-A (39). Thus, these cells are an excellent *in vitro* model for gonadotroph function. Twenty-four hours before the transient transfection experiments, L β T2 cells were plated in 24-well trays (1.25 \times 10⁵ cells/well) and grown in DMEM/F-12 media supplemented with 10% FBS. A total of 300 ng cDNA, consisting of 150 ng pARE-GLP-lux, 120 ng pFAST-1, and 30 ng pRL-TK (Promega Corp.) were complexed to 3 μ l Effectene for each well as per manufacturer's instructions. The cells were then transfected with the DNA/lipid complexes for 16 h at 37 C. The media were then replaced with serum-free DMEM/F-12 containing 0.1% BSA supplemented with FS288 (ranging from 2.5 ng/ml to 100 ng/ml) or FSRP Fc (ranging from 5 ng/ml to 150 ng/ml). After treating for 24 h, the cells were lysed and assayed for luciferase activity. Interwell variations in transfection efficiency were corrected by normalizing to Renilla using a dual-luciferase reporter assay (Promega Corp.).

Heparin-binding assays

To test for FSRP binding to heparin sulfate, we used a sulfate cellulose affinity matrix (Amicon, Danvers, MA), which has been used extensively to characterize FS288 (31). FS288 or FSRP-Fc (30 ng), BSA (100 ng), or nothing was added to tubes containing RIA buffer (0.01 M PBS, 0.1% gelatin, 0.05% Tween-20) up to 200 μ l total volume. Radiolabeled activin (20 μ l, 200,000 cpm) was then added and the samples incubated at 20 C for 2 h. To precipitate activin complexed to heparin-binding proteins, 30 μ l of a 50% slurry of sulfate cellulose was added and the tubes incubated for another 2 h. At the end of this incubation, the tubes were centrifuged at 5000 \times g for 5 min, the supernatant aspirated, RIA buffer added and

the pellet resuspended. After centrifugation, the supernatant was aspirated and the pellet was counted in a γ counter.

To examine FSRP binding to cell surface proteoglycans, HEK 293 cells were plated in 6-well trays. When 50–70% confluent, the cells were washed and medium replaced with 1 ml assay medium consisting of DMEM + 0.1% BSA. FS288 or FSRP-Fc (30 ng) along with a control containing medium alone were added to two wells each. A duplicate set of wells were treated with 10 μ g/ml heparan sulfate (Sigma, St. Louis, MO) to remove FS complexes bound to cell surface proteoglycans. After a 2-h incubation, the medium was aspirated and the cells washed, fresh assay medium containing 125,000 cpm radiolabeled activin was added, and the cells incubated at 20 C for an additional 4 h. At the end of the incubation, the cells were washed twice and solubilized in 1 N NaOH for 15 min. The supernatant was transferred to RIA tubes and counted in a γ counter.

Results

N-terminal sequencing of recombinant human FSRP

Based on the high degree of structural and primary sequence conservation between FSRP and FS, we first hypothesized that the cleavage site of FSRP's signal peptide would, like FS, be located at the first exon/intron junction (35). To test this, we sequenced purified, recombinant FSRP-Fc and found that the first residue is actually methionine at position 26 relative to the translational start site. Thus, signal peptide cleavage in FSRP actually precedes the exon/intron boundary for exon 1 by nine residues. When compared with the amino acid sequence of FS, the N-terminal domain in FSRP is extended by an additional nine residues (Fig. 1) so that signal peptide cleavage actually occurs within exon 1, a potentially important difference with FS.

Ligand specificity of FSRP and FS

To directly assess the relative specificity of FSRP and FS for binding various members of the TGF- β superfamily, compared with activin-A, we used a previously developed solid-

phase radioligand-binding assay in a nonequilibrium format (4) because of the nonreversible nature of activin-FSRP (35) or FS (4) complexes. As shown in Fig. 2, increasing doses of unlabeled activin inhibited binding of radiolabeled activin with ED₅₀ values of approximately 3.2 and 0.8 ng/well for FSRP and FS, respectively. In contrast, although BMP-7 was able to compete with labeled activin for binding to both FS and FSRP, the relative potency of this closely related TGF- β family member was approximately 500- to 1000-fold lower. BMP-4, inhibin-A, MIS, and TGF- β ₁ had no detectable inhibitory activity in this assay. Thus, FSRP and FS have a similar preference for activin-A within the TGF- β superfamily, the difference in ED₅₀ for unlabeled activin between FSRP and FS being consistent with our previously estimated 2.4-fold higher affinity of FS for activin (35).

FSRP and FS inhibit activin binding to its receptor

It has been previously shown that FS can prevent activin from binding its receptor, thereby accounting for at least some of its ability to inhibit activin's actions (5). We therefore examined whether FSRP shared this activity using HEK 293 cells that were transiently transfected with hActRII cDNA to enhance activin binding, a procedure that increased activin binding almost 2.5-fold over basal, and more than 4-fold over nonspecific binding (Fig. 3). In this system, both FSRP and FS were able to bind radiolabeled activin and inhibit its access to hActRII, with FS being approximately 2-fold more potent than FSRP-Fc, consistent with the 2.4-fold higher affinity of FS for activin, compared with FSRP-Fc (34). These results are consistent with a common mechanism for FS and FSRP inhibition of activin in solution. A similar pattern was observed when COS 7 cells were tested under the same conditions (data not shown).

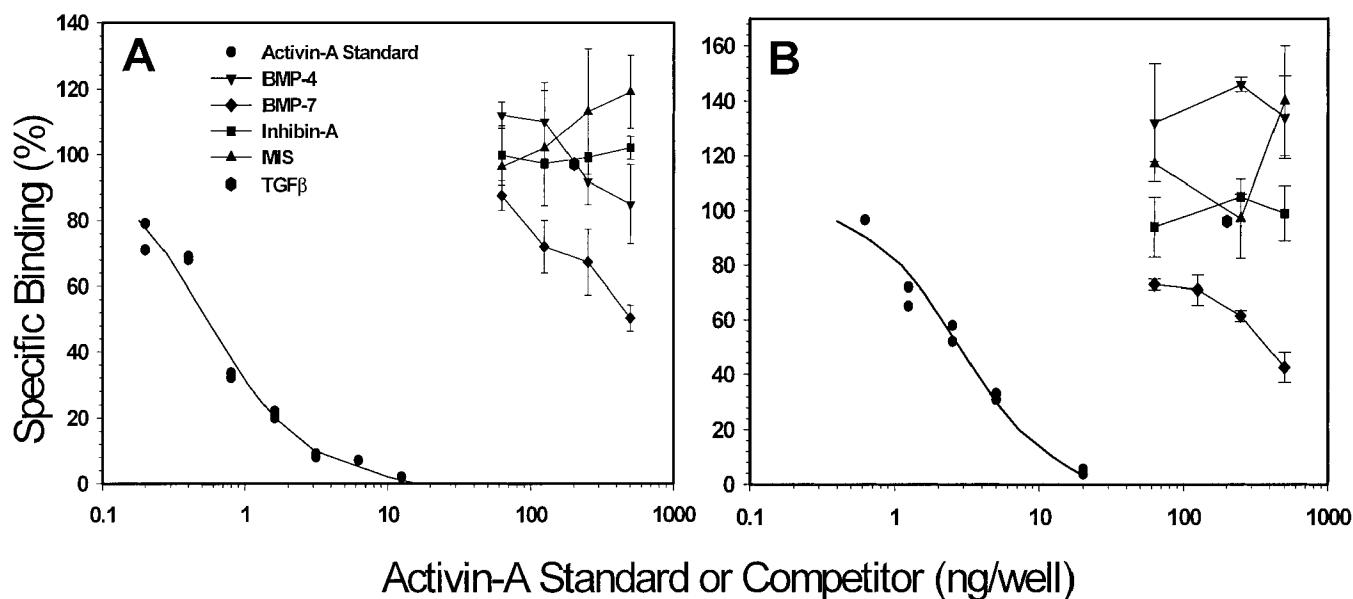


FIG. 2. Ligand specificity of FS and FSRP—nonequilibrium competition assay. Increasing doses of activin or related members of the TGF- β superfamily were added to FS (A) or FSRP (B) coated wells. After 1-h preincubation, radiolabeled activin was added for an additional hour. Of the ligands tested, only BMP-7 showed detectable competition with labeled activin for binding to either FSRP or FS but at 500- to 1000-fold lower potency than activin itself. Inhibin-A, MIS, TGF- β ₁, and BMP-4 were not active in this assay. Shown are results from one of three representative assays.

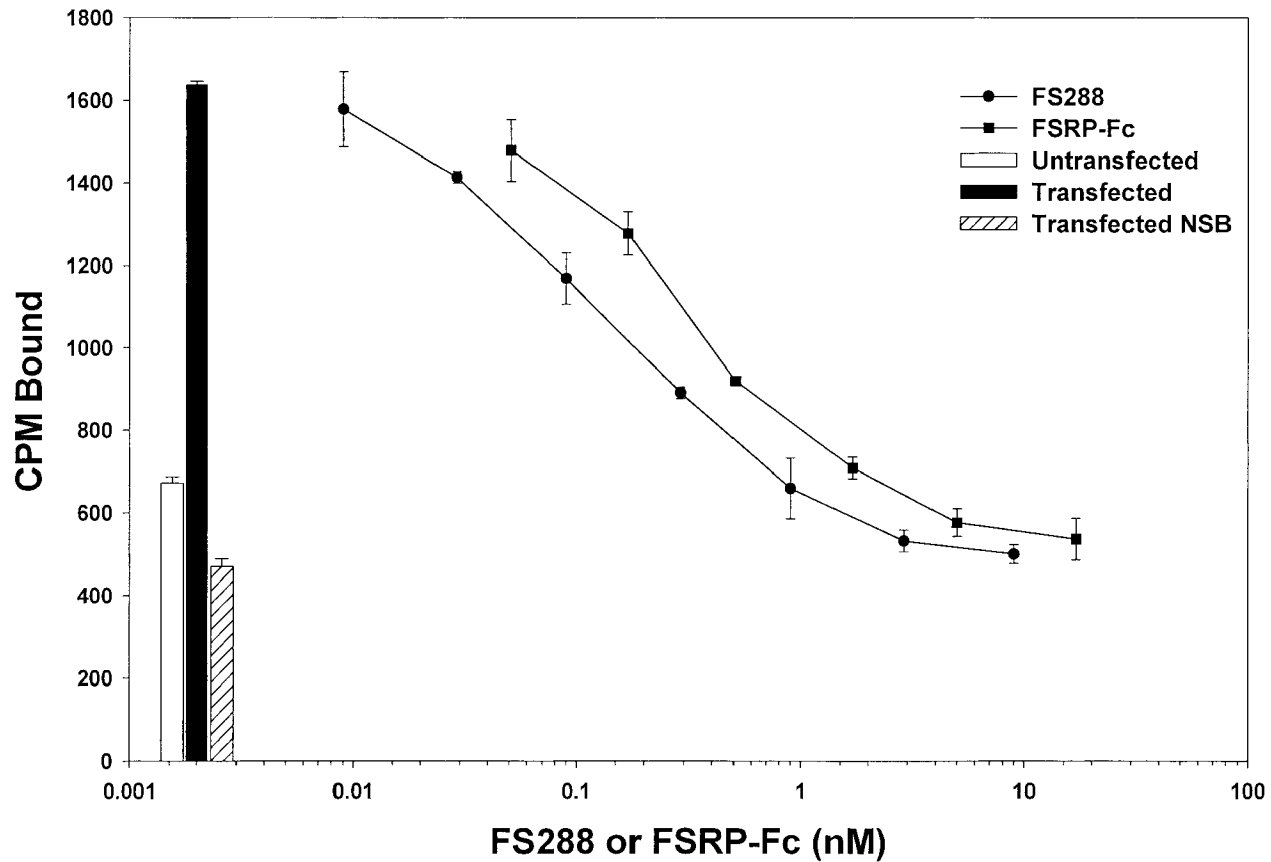


FIG. 3. Inhibition of activin's access to its receptor. Activin complexed to either FS or FSRP was incubated with HEK 293 cells transiently transfected with human ActRIIA cDNA. The ED_{50} for FSRP's inhibition of activin binding to its receptor is approximately 2-fold higher than that of FS288, a difference consistent with the differential affinity of these two proteins for activin binding (35). Shown are results from one of three representative experiments.

FSRP and FS inhibition of endogenous activin activity

Follistatin's biological activity was originally defined on the basis of its ability to suppress FSH secretion from anterior pituitary cell monolayer cultures, an effect mediated by its ability to neutralize endogenous activin produced by gonadotropes (11). We therefore examined the potency of FSRP relative to FS in this assay. As expected, FS was able to inhibit FSH secretion from rat gonadotropes at subnanomolar doses (Fig. 4). Surprisingly, neither FSRP-Fc nor FSRP-FLAG-suppressed activin-mediated FSH biosynthesis in this assay at concentrations nearly 50-fold greater than the minimally effective concentration of FS (~ 0.25 nM). The FSRP used for this bioassay was confirmed to be biologically active because aliquots from the identical vial used contemporaneously were able to prevent activin from binding to its type II receptor (Fig. 3) and stimulating transcription (see Fig. 6).

To investigate this apparent paradox further, we tested the relative ability of FSRP-Fc and FS to inhibit activin-mediated gene transcription in the L β T2 mouse pituitary cell line. These cells were previously shown to secrete FSH under the influence of exogenous activin, produce abundant activin- β B message, and synthesize FSH β mRNA in response to endogenously produced activin, an effect that can be inhibited by FS (39). As expected, the endogenous activin produced by these cells resulted in relatively high basal reporter activity

that was inhibited dose dependently by FS288; the maximal inhibition being observed at 0.8 nM FS (Fig. 5). In contrast, FSRP-Fc was nearly 100-fold less potent at inhibiting reporter gene activation by endogenous activin. Thus, in contrast to their similar ability to inhibit activin binding to its receptor, these two binding proteins are quite different in their ability to neutralize endogenous activin in primary gonadotropes or a gonadotrope cell line, an effect that cannot be accounted for by the 2.4-fold difference in activin-binding affinity.

Inhibition of exogenous vs. endogenous activin in an HEK 293 cell model

To directly compare the differential neutralization activity of FSRP and FS on endogenously or exogenously produced activin, we used our previously characterized activin transcriptional reporter system (32) in HEK 293 cells, which contain all of the necessary activin-signaling components. In the absence of added activin, reporter activity is low but increased nearly 5-fold on stimulation with 0.2 nM activin-A (Fig. 6). In this assay system, both FS and FSRP were able to suppress activin-stimulated reporter activation with approximately 3-fold difference in potency, consistent with the 2.4-fold higher affinity of FS for activin. Thus, both FS and FSRP can inhibit the activity of exogenously added activin-A.

To examine the effects of FS and FSRP on endogenous

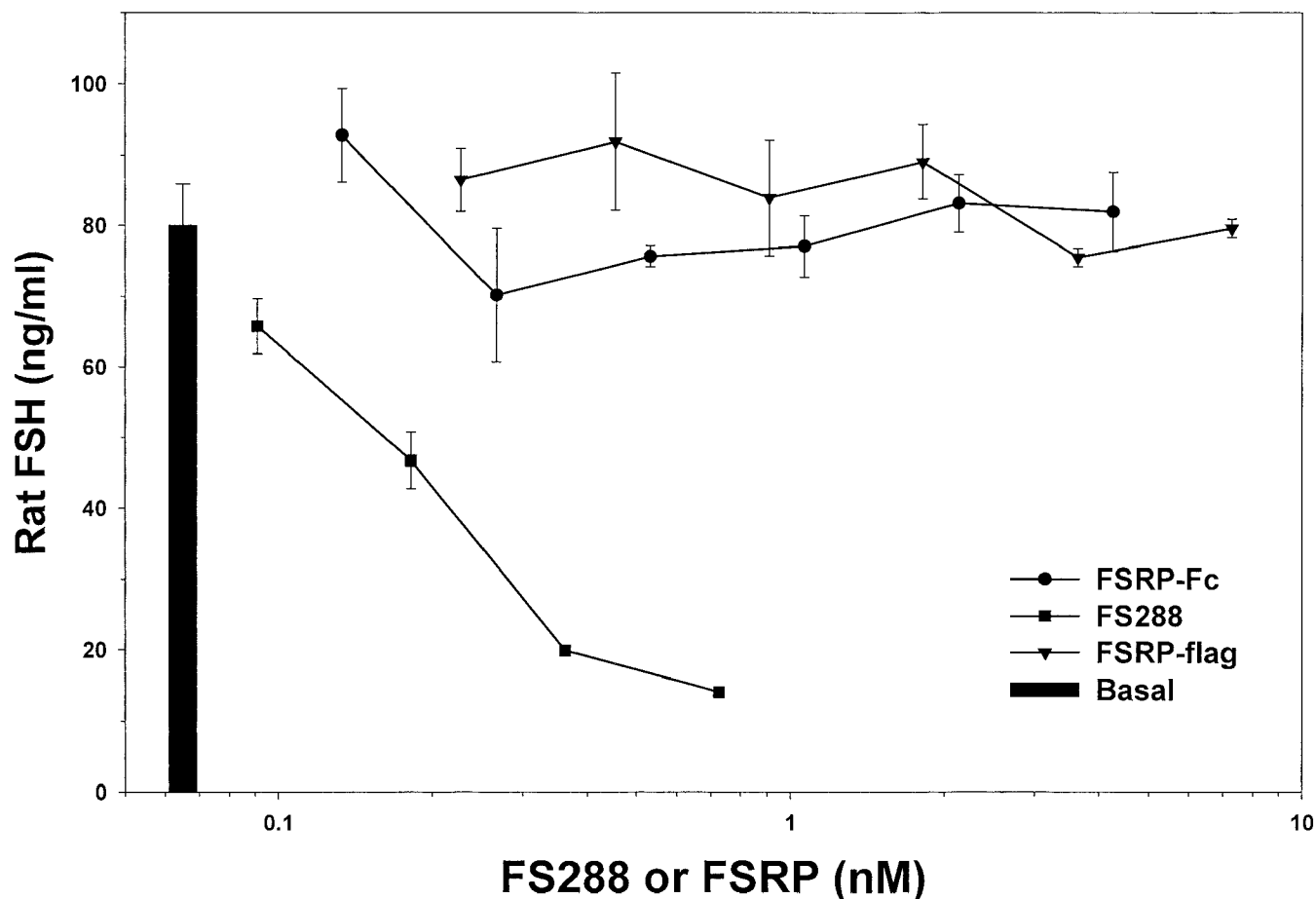


FIG. 4. FSRP did not repress the FSH secretion induced by endogenous activin in the rat pituitary bioassay. Rat anterior pituitaries were dispersed and cultured in the presence of either FS or FSRP. FS inhibited FSH secretion from rat anterior pituitary cells in a dose-dependent manner over a range of 0.1–0.5 nM. However, neither highly purified recombinant FSRP-FLAG nor FSRP-Fc was active in this assay at concentrations nearly 50-fold higher than the minimally effective concentration of FS. These same preparations of FSRP were active in receptor binding and activin-binding assays. Shown are results from one of three representative experiments.

activin in this system, activin- β A or - β B subunit cDNA was cotransfected with the reporter, resulting in robust stimulation of reporter activity to levels comparable with those achieved by exogenous activin (Fig. 7). FS inhibited this basal activin-A (Fig. 7, *top*) or activin-B (Fig. 7, *bottom*) activity in a dose-dependent manner as observed in the primary rat pituitary cells (Fig. 4) or L β T2 cell line (Fig. 5). In contrast, FSRP inhibited basal reporter activity by less than 20% at doses at least 100-fold higher than those used for FS for the same level of inhibition. Taken together, these results indicate that FSRP can inhibit exogenous activin-A with similar potency to FS but is more than 100-fold less active than FS when the activin (A or B) is derived endogenously.

Inhibition of exogenous vs. endogenous activin in primary pituitary cells

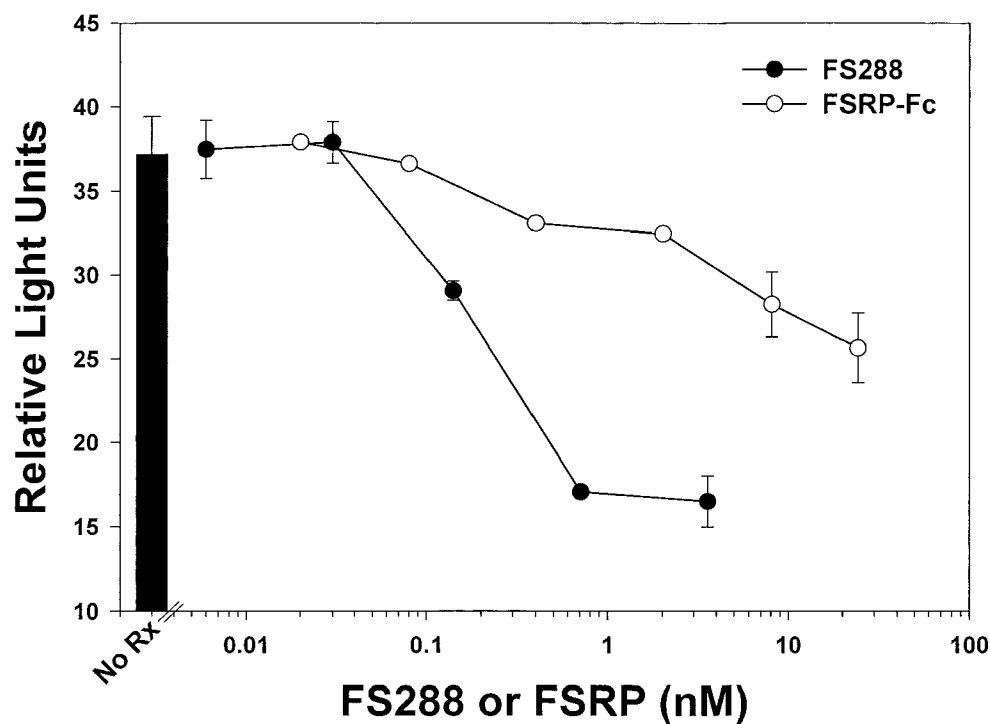
To confirm these observations in a more physiological context, the neutralization activity of FS and FSRP were compared using dispersed rat pituitary cultures in the presence or absence of exogenous activin-A. As expected, in the absence of exogenous activin, FS inhibited FSH release at subnanomolar doses, and FSRP was inactive at more than

100-fold molar excess concentrations. However, in the presence of 0.35 nM activin-A, a dose that maximally increased FSH release by about 35%, both FS and FSRP inhibited the exogenous activin-mediated FSH secretion (Fig. 8). Moreover, although higher doses of FS continued to inhibit FSH release mediated by endogenous activin, FSRP was unable to modulate endogenous activin. These results therefore demonstrate that even in primary cells, FSRP, in stark contrast to FS, is able to neutralize activin from endocrine or paracrine sources but not autocrine-derived activin.

Binding to heparin sulfate or cell surface proteoglycans

FS288 can bind to cell surface heparin-sulfated proteoglycans (30), under some conditions forming a barrier around cells such that exogenous activin is prevented from accessing its receptor (32). The absence of a consensus heparin binding sequence in FSRP (Fig. 1) suggests that, unlike FS, FSRP will not bind heparin or to cell surface heparin-sulfated proteoglycans, perhaps accounting for its reduced ability to neutralize endogenous activin. As expected, FSRP was unable to bind to a negatively charged heparin-like matrix above the levels attained by the buffer or BSA controls, whereas FS288

FIG. 5. FSRP weakly repressed signaling induced by endogenous activin in L β T2 cells. L β T2 cells transfected with an activin reporter system show a basal level of luciferase activity that is potently inhibited by FS288 administration. FSRP's activity in this assay is approximately 100-fold lower than similar doses of FS, further demonstrating that FSRP is a relatively weak inhibitor of endogenous activin activity. Shown are results from one of three representative experiments.



Exogenous Activin-A

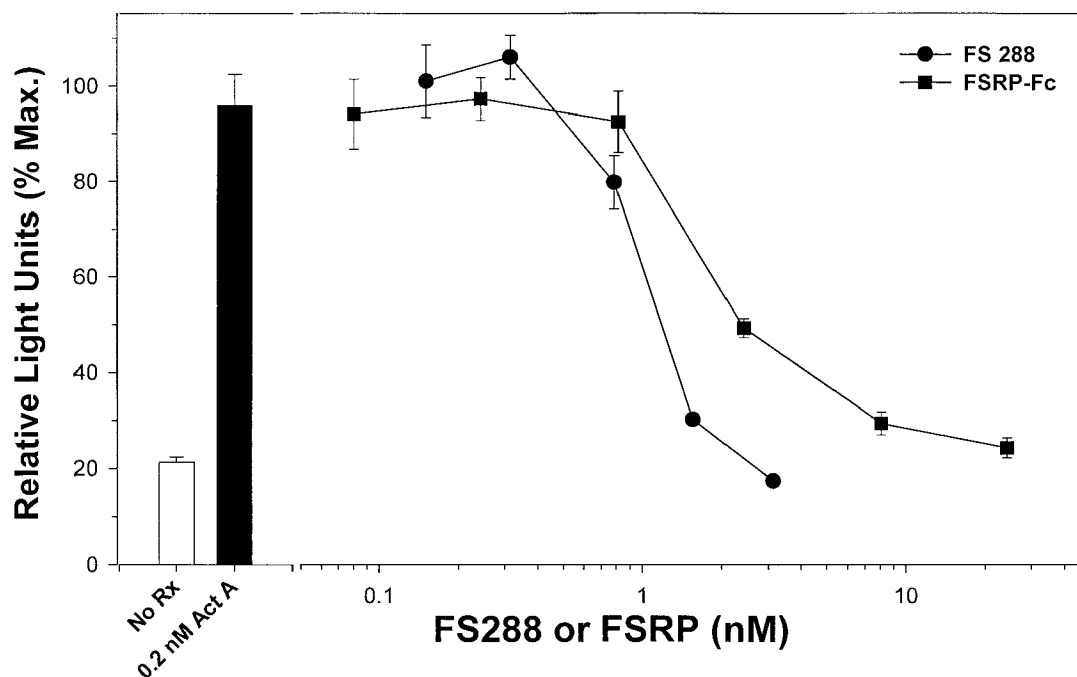


FIG. 6. FSRP and FS repressed signaling induced by exogenous activin in HEK 293 cells. Activin-A treatment (0.3 nM) of HEK 293 cells transiently transfected with an activin-responsive luciferase reporter system resulted in nearly 5-fold higher signal transduction over basal, and this effect was inhibited by increasing concentrations of FS288, reaching maximum at about 3 nM. FSRP-Fc was about approximately 2-fold less potent than FS288 in this assay, consistent with the 2.4-fold difference in their relative affinities for activin (26). Shown is the mean (\pm SE) for three experiments.

binding was 4-fold greater than controls (Fig. 9A). Similarly, FSRP was unable to bind to cell surface proteoglycans on cultured HEK 293 cells, whereas FS288 binding was 6-fold greater than control wells (Fig. 9B). In both cases, FS288 or

FSRP binding was determined using labeled activin, and we confirmed that the activin was bound to FS288 on the cell surface by the addition of 10 μ g/ml heparin sulfate, which released the FS-activin complex (40). Thus, the inability of

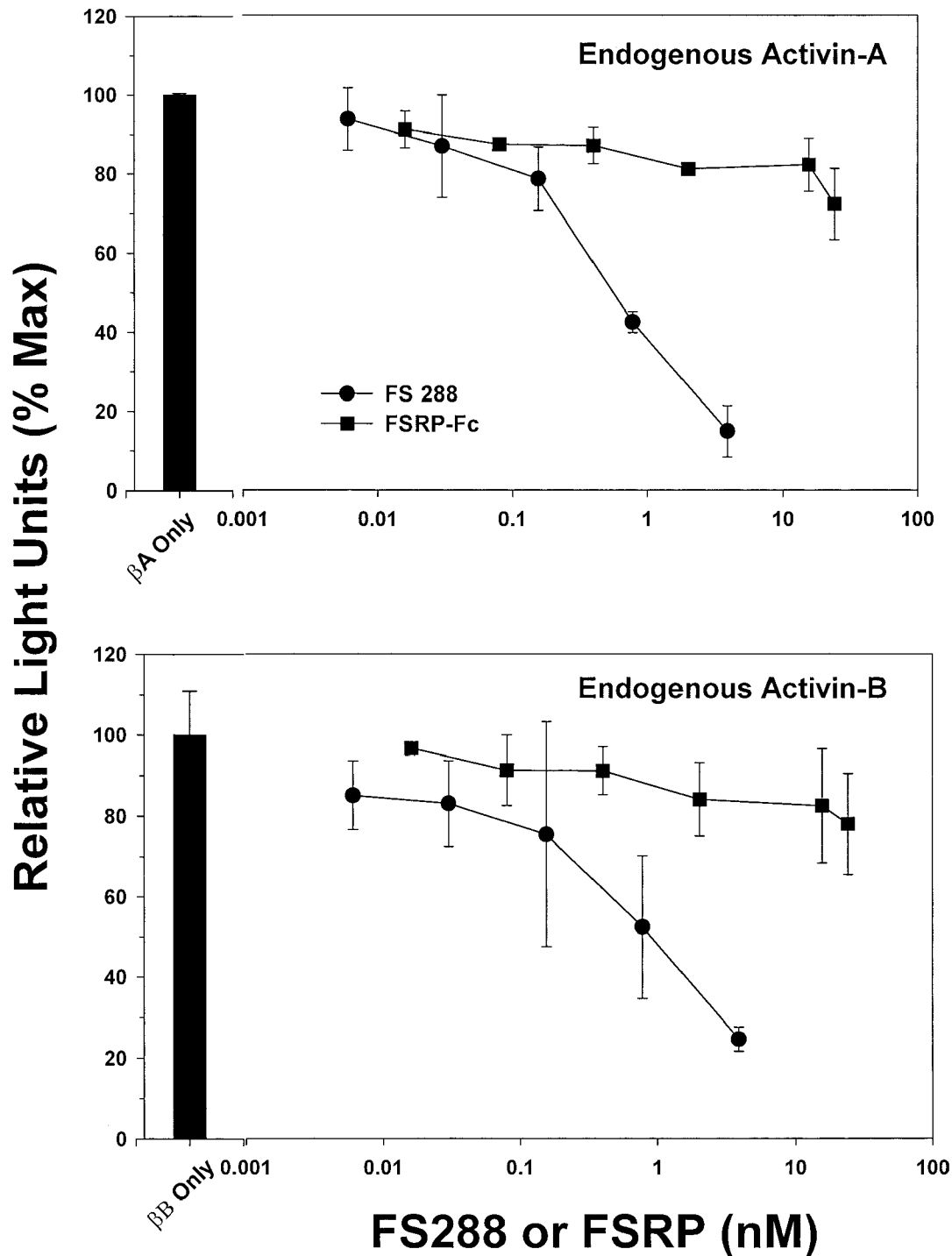


FIG. 7. FSRP weakly repressed signaling induced by endogenous activin-A or -B in HEK 293 cells. HEK 293 cells were transfected with the activin reporter system as well as either activin- β A (*top*) or - β B (*bottom*) subunit cDNA. In both cases, activin-mediated signal transduction was increased 5- to 10-fold. FS288 inhibited signaling induced by either activin-A or activin-B in a dose-dependent manner over a range of 0.1–5 nM, but FSRP showed little or no inhibition at more than 25-fold higher doses. Shown is mean (\pm SE) of three experiments.

FSRP to bind cell surface proteoglycans may account for its reduced potency in neutralizing endogenous activin activity.

Discussion

Although it is clear from previous studies that FSRP and FS share many structural features, the degree to which their

functions overlap remains to be elucidated. Toward this end, we have now examined and compared the activity of these proteins in a variety of cellular systems and found that despite their common ability to bind activin with high affinity, they differ significantly in their ability to neutralize activin derived from endogenous sources, raising the exciting pos-

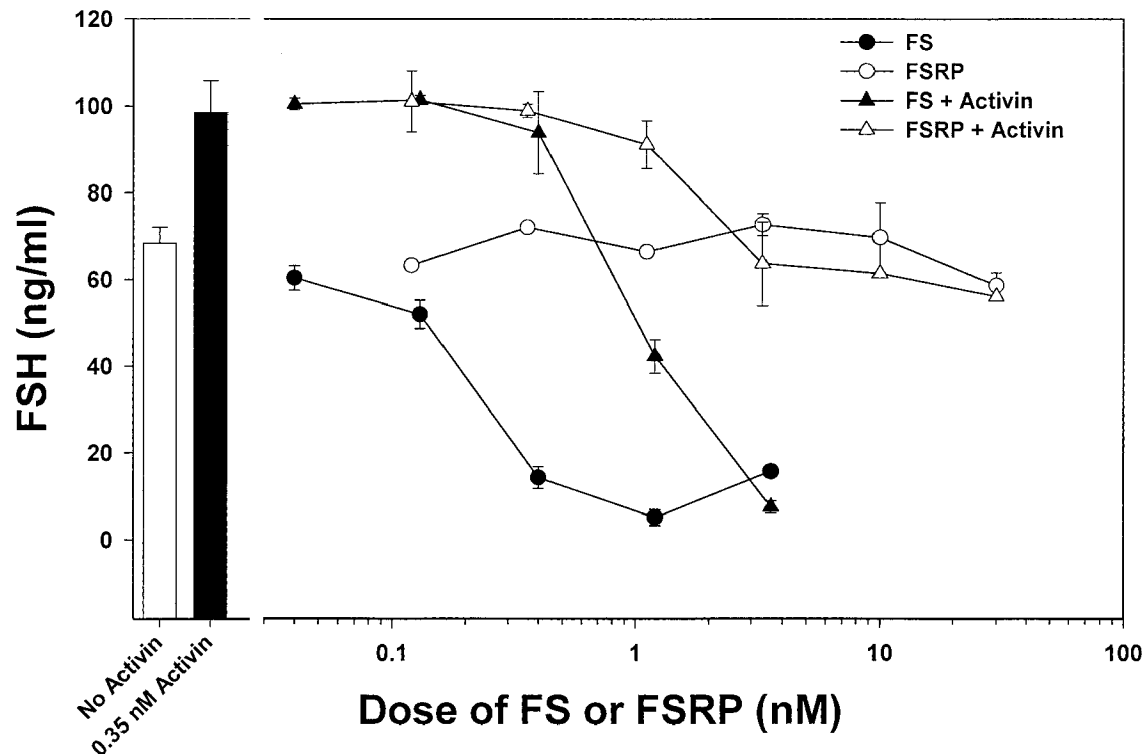


FIG. 8. Neutralization of activin by FS and FSRP in the primary rat pituitary assay. In the absence of added activin, FSRP had no activity, but FS inhibited endogenous activin at subnanomolar doses (compare *open* and *closed circles*). Addition of 0.35 nM activin-A, the maximally effective dose in this assay, increased FSH secretion by approximately 35% over basal. This stimulation by exogenous activin could be inhibited by both FS and FSRP (compare *open* and *closed triangles*). However, FSH secretion because of endogenous activin was not blocked by FSRP, even at doses up to 100-fold greater than the minimally active FS dose (compare highest three *open triangle doses* to *closed triangles*), confirming observations in HEK 293 cells and indicating potential differential bioactivity for FSRP and FS. This figure shows one of three representative experiments.

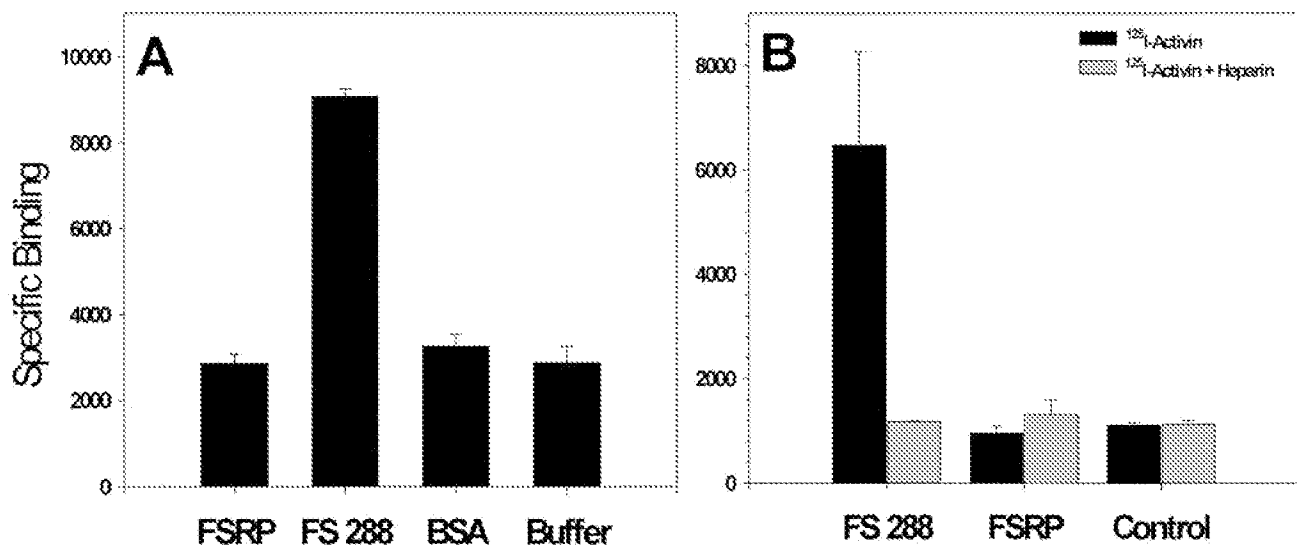


FIG. 9. FSRP does not associate with heparin-like matrix or cell surface proteoglycans. A, Binding to sulfate cellulose. Radiolabeled activin was added to tubes containing buffer, with or without BSA (nonspecific protein), FSRP, or FS. After 2 h, binding to sulfate cellulose, a heparin sulfate-like matrix, was assessed. FS288 bound to the matrix but FSRP binding was not different from controls, consistent with the lack of a heparin-binding domain in FSRP. B, Binding to HEK 293 cells. FSRP, FS, or BSA was added to culture HEK 293 cells for 2 h, the medium changed, and radiolabeled activin added for an additional 4 h. FS288-activin complexes bound well to cell surface proteoglycans, whereas FSRP-activin complexes did not bind above control levels. Heparin sulfate (10 μ g/ml) inhibited FS288 binding to cell surface heparin-sulfated proteoglycans as expected but had no effect on FSRP or control cells. Taken together, these studies confirm that the absence of a heparin-binding motif in FSRP likely precludes its binding to heparin or cell surface proteoglycans like FS. Shown are results from one of three representative experiments.

sibility that FSRP and FS have nonoverlapping cellular functions.

The striking homology in primary sequence, exon/intron arrangement, and domain structure previously identified between FSRP and FS supports the hypothesis that they have overlapping functions, perhaps predominating in different tissues or at different developmental stages. Moreover, FSRP, like FS, binds activin with high affinity and nearly irreversible kinetics and prefers activin over its close TGF- β superfamily relatives BMP-6, BMP-7 (35), and BMP-2 (36). Finally, it was observed that FS plays a key role in *Xenopus* embryonic neuralization (19), yet the FS knockout mouse did not show significant neural defects (41). Because both FS and FSRP mRNA are expressed in the brain (35, 36), it is possible that FSRP is able to functionally compensate for FS in the FS knockout mouse.

To explore further the degree of functional overlap of these two proteins, we first employed a solid-phase competitive binding assay previously used to characterize FS (4) to more completely characterize the relative specificity of FSRP and FS for activin and related ligands. Both FSRP and FS selectively bound activin, with the closely related BMP-7 (42) binding with approximately 500-fold lower potency. Inhibin A, MIS, TGF- β 1, and BMP-4 did not bind to either FS or FSRP. This agrees well with our previous demonstration of FSRP and FS directly binding radioiodinated activin, BMP-6, and BMP-7, with the latter ligands being at least 20- to 40-fold less active (35). Direct binding and inhibition of BMP-2 activity by FSRP and FS was also previously demonstrated (36), although the preparations of FSRP were only partially purified so comparisons of relative affinity between the two binding proteins could not be directly assessed. In addition, the tryptophan residues at positions 4 and 36 of FS, previously shown to be critical for activin binding (26), are conserved in FSRP, suggesting that structural determinants of activin binding are preserved between the two molecules. Thus, the present data suggest that the determinants of ligand specificity and binding are also preserved between FSRP and FS.

On the other hand, a number of differences between FSRP and FS have been identified that suggest the presence of nonoverlapping roles in regulating activin action. We recently demonstrated that FSRP, unlike FS, can be found in the nucleus of many cell types; moreover, it appears to be secreted only at expression levels higher than that required for its immunocytochemical detection within the nucleus (35). Although potential binding partners and nuclear actions of FSRP remain to be defined, this difference in intracellular trafficking suggests that FSRP may have biological roles that are quite distinct from those attributable to FS. The observations in the present study that FSRP signal peptide cleavage results in an N-terminal extension of the critical activin-binding domain relative to FS and the greatly reduced ability of FSRP to neutralize endogenously produced activin further support this concept.

In fact, the nine-residue N-terminal extension of the N domain of FSRP is important from at least two standpoints. Because there appears to be no classic nuclear localization signal elsewhere within FSRP, this extension conceivably

could influence protein trafficking. Furthermore, our results show that the addition of amino acids to the N-terminal region of FS may not be as detrimental to activin binding as was previously believed (43). Despite the nine additional amino acids in its N-terminal domain, FSRP has an affinity for activin quite comparable with that of FS (35).

In the present study, we used purified preparations of FS and FSRP to directly compare their activin-neutralizing activities in a variety of potentially physiological circumstances. When we examined situations in which activin might arrive from outside the responding cell, such as activin derived from paracrine or endocrine sources, the relative inhibitory activities of FSRP and FS for activin binding to its receptor and the neutralization of activin-stimulated gene transcription were compatible with the 2.4-fold difference in their affinities for activin (35). These results agree with the recent demonstration that relatively high doses (0.1–1 μ g/ml) of a partially purified mouse FSRP preparation suppressed activin-A-stimulated signal transduction in K562 and CHO cells. Unfortunately, this activity was not compared with FS so relative activities could not be directly assessed (36).

On the other hand, when we compared the activin-neutralizing ability of FSRP and FS for activin derived from the responding cell, as with autocrine-derived activin, FSRP was clearly much less potent. For example, in the primary rat pituitary cell bioassay in which FS was originally defined and activin was produced by the gonadotrophs themselves, FSRP was still inactive at concentrations nearly 100-fold higher than the minimal effective concentration of FS. This observation was confirmed using a mouse pituitary cell line (L β T2) in which endogenous activin-mediated reporter transcription is neutralized to a much greater degree by FS, compared with FSRP. Because our reporter system was maximally stimulated in these cells, even in the absence of exogenous activin, we could not use these cells to compare the effects of FSRP and FS on activin derived from different sources.

Thus, we used two approaches to investigate the differential regulation of FSRP and FS for exogenous *vs.* endogenous activin in the same *in vitro* assay. First, in a cellular system in which the dose of exogenous and endogenous activin could be controlled, we employed HEK 293 cells, which contain a functional activin signaling system but do not produce detectable activin. When the cells were treated with exogenous activin, both FSRP and FS were able to neutralize activin-mediated gene transcription. However, if the cells were cotransfected with β A cDNA to produce endogenous activin-A, FSRP was more than 50-fold less potent than FS. Moreover, when β B cDNA was transfected to produce endogenous activin-B, FS had similar neutralizing activity to that observed for activin-A, but FSRP was nearly inactive even at 100-fold higher concentrations. The second system used primary rat pituitary monolayer cultures in the presence or absence of exogenous activin. In this case, exogenous activin was neutralized by both FS and FSRP with the expected relative activity. However, higher doses of FS neutralized endogenous activin, whereas FSRP was unable to do so up to 100-fold molar excess over the minimally active FS dose. Taken together, these results confirm our observation

that FSRP and FS differentially neutralize exogenous *vs.* endogenous activin. Moreover, they demonstrate that FS is capable of neutralizing both activin-A and activin-B with similar potency. Because many activin-responsive tissues also produce activin (12, 44–46), our results suggest the strong possibility that the physiological activities of FSRP and FS do not entirely overlap.

At least one mechanism whereby FS inhibits activin action involves binding to cell surface heparin-sulfated proteoglycans (40), which is mediated by the heparin binding sequence located in FS domain 1 (Fig. 1). One consequence of the ability of FS to bind cell surface proteoglycans is to form a barrier, at least at high concentrations, to the biological activity of exogenous activin, as recently demonstrated in the FS288-secreting PA-1 teratocarcinoma cell line (32). Interestingly, in these same cells, this FS barrier had no effect on endogenous activin (32). In the present studies, however, exogenous FS was able to modulate endogenous activin, both in the rat pituitary and in the L β T2 cell transcriptional assay. This difference may be due to the level of activin production relative to the FS concentration outside the cell or may indicate that in some cells, activin can exert its biological action(s) before it is secreted and thus exposed to FS.

FSRP does not have a heparin-binding sequence, and our studies are a strong indication that its absence prevents cell surface association of FSRP. Thus, it is possible that FSRP is unable to achieve the local concentration in the region of the cell surface activin receptor that FS can achieve, thereby leading to FSRP's reduced ability to neutralize endogenous activin signaling although it retains similar activity to FS in neutralizing exogenous activin. In any event, our results suggest that for exogenous activin, the relative ability of extracellular FSRP and FS to neutralize its actions depend on their relative affinities for activin and their concentrations relative to activin. On the other hand, the ability to neutralize endogenous activin appears to depend largely on the ability of FS to associate with the cell surface and bind activin before it has an opportunity to access its type II receptor, an action not available to FSRP. Although mutational analysis of FS clearly correlates activin binding with biological activity (26), the distinctions between FS and FSRP described here suggest that activin-binding activity is necessary but not sufficient to account for all of the actions of FS. Future investigation using site-directed mutagenesis and domain-swapping strategies between FSRP and FS will likely shed additional light upon structure-function relationships for these closely related proteins.

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